ELSEVIER

Biochimica et Biophysica Acta 1232 (1995) 81–88

BBA Biochimica et Biophysica Acta

Identification of a photochemically inactive pheophytin molecule in the spinach D_1-D_2-cyt b_{559} complex [1]

Mamoru Mimuro [a,*], Tatsuya Tomo [b,c], Yoshinobu Nishimura [d], Iwao Yamazaki [d], Kimiyuki Satoh [b,c]

[a] *Division of Bioenergetics, National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan*
[b] *Division of Biological Regulation, National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan*
[c] *Department of Biology, Faculty of Science, Okayama University, Okayama 700, Japan*
[d] *Department of Chemical Process Engineering, Faculty of Engineering, Hokkaido University, Sapporo 060, Japan*

Received 12 January 1995; revised 6 July 1995; accepted 17 July 1995

Abstract

A photochemically inactive pheophytin (Pheo) *a* molecule in the spinach D_1-D_2-cyt b_{559} complex, which contains approximately 6 chlorophyll (Chl) *a* molecules and two β-carotene molecules for every two Pheo *a* molecules, was identified by fluorescence excitation spectroscopy at 77K, and confirmed by absorption, linear dichroism (LD), and magnetic circular dichroism spectra. When the emission was monitored at 740 nm, photochemically active Pheo *a* was detected as peaks at 418, 513, 543, and 681 nm, as described in previous reports. However, when the emission was monitored at 665 nm, two bands were observed at 414 and 537 nm; these bands were insensitive to photochemical reduction of the primary acceptor and thus appear to be due to photochemically inactive Pheo *a*. The *Qy* band of this Pheo *a* molecule at 77K was estimated to be at 671 ± 1 nm, based on the magnitude of the band-shift in photochemically active Pheo *a*. Two β-carotene molecules in the complex were also discriminated by fluorescence excitation and LD spectra. These observations were discussed in the light of current understanding of the molecular organization of pigments and the relationship of pigment arrangement to optical properties in the reaction center of photosystem II.

Keywords: Fluorescence; Pheophytin; Photosynthesis; Reaction center II; Spinach

1. Introduction

The molecular arrangement of prosthetic groups in the reaction center (RC) of photosystem (PS) II is considered to be analogous to that of purple bacterial RC, based on similarities between the primary structure of the D_1 and D_2 polypeptides and that of the L and M subunits [1,2]. Because of these similarities, the mechanism of electron transfer in the purple bacterial RC has often been applied to the RC of PS II [1–3]. However, several reports have described differences in the molecular arrangement of special pairs [4–6] and other pigments [7–9] between these two types of RC. Thus, analysis of pigment organization in the PS II RC is crucial to our understanding of the primary processes of energy conversion. The application of various types of spectroscopy is particular important at this stage of analysis, as the three-dimensional structure of PS II RC has yet to be resolved.

The stoichiometry of prosthetic groups in PS II RC preparations isolated in the previous studies has varied to some extent [10–14], but the basic constituents are Chl *a*, Pheo *a*, β-carotene, and cyt b_{559}; the quinone molecule is absent [10]. The relative content of Chl *a* and β-carotene per two Pheo *a* molecules ranges from four to six and from one to two, respectively [10–14]. Thus it appears that the PS II RC complex may contain one or two more Chl *a* molecules and possibly one more β-carotene molecule than the purple bacterial RC, although the localization and

Abbreviations: (B)Pheo, (bacterio)pheophytin; (B)Chl, (bacterio)chlorophyll; cyt, cytochrome; DF, delayed fluorescence; LD, linear dichroism; MCD, magnetic circular dichroism; PS, photosystem; P680, primary electron donor in reaction center II; P680*, the excited state of P680; RC, reaction center.

* Corresponding author. Fax: +81 564 53 7400; e-mail: mimuro@sesame.nibb.ac.jp.

[1] This paper is dedicated to Prof. Yoshihiko Fujita, National Institute for Basic Biology, on the occasion of his retirement.

SSDI 0005-2728(95)00116-6

function of the additional molecules are unclear. Differences in pigment composition are inevitably reflected in the optical properties of a complex. For example, the deconvolution of absorption spectra indicates that the *Qy* transition of Chl *a* and Pheo *a* is located within the region of 665 to 683 nm [7,9,15–22]. However, specific pigments cannot always be described by unique absorption bands; P680 is reported to be located at 680 nm [9,15–22], whereas the absorption maximum of photochemically active Pheo *a* ($Pheo_{ac}$) has been reported at 672 nm [17], 676 nm [9,15], 680 nm [19], and 682 nm [10,14,20]. In contrast, a hole-burning experiment indicated that the energy difference between Pheo *a* and P680 was 25 cm^{-1} at 4.2 K [21]. Although considerable research has been done on some of the pigments in PS II RC, other pigments, such as accessory Chl *a* and photochemically inactive Pheo *a* ($Pheo_{iac}$), have not yet been linked to specific absorption bands. In the present study we used fluorescence spectroscopy at 77 K as a major tool to determine the location of $Pheo_{iac}$ in D_1-D_2-cyt b_{559} complex isolated from spinach. We also discuss how the arrangement and optical properties of prosthetic groups relate to the mechanism of primary photoreactions in the RC complex.

2. Materials and methods

The D_1-D_2-cyt b_{559} complex was isolated from spinach grana thylakoids by the previously described method [10], with a minor modification; after the complex was isolated, we replaced Triton X-100 with digitonin (0.2%) by the following method [23]. The samples were adsorbed to a small DEAE-Toyopearl 650S (Toyo Soda, Japan) column (1.2 × 2.0 cm), washed with 100 ml of Mes-NaOH buffer (50 mM, pH 6.5) containing 0.2% digitonin, and then eluted with 10 ml of the same buffer containing 500 mM NaCl and 0.2% digitonin. When necessary, the sample was concentrated with ultrafiltration and dialyzed against Mes-NaOH buffer (50 mM, pH 6.5) containing 0.2% digitonin. The samples were suspended in the same buffer and spectral measurements were conducted. Photochemically reduced Pheo *a* was trapped by adding sodium dithionite (2 mg/ml) and methyl viologen (2 μM) to the sample solution, illuminating the solution for 3 min at approx. 0°C with white light from an incandescent lamp (4500 $\mu E/m^2/s$), and then freezing the sample while still under illumination. Pigment content was determined by HPLC analysis of the acetone-extracted samples [12], and was estimated to be 6.23 ± 0.26 and 2.18 ± 0.24 for Chl *a* and β-carotene, respectively, per 2 Pheo *a* molecules. Extinction coefficients of Chl *a* and Pheo *a* were reported by Watanabe et al. [24] and of β-carotene, by Davies [25], respectively.

Absorption and fluorescence spectra at 77 K were measured with a Hitachi 557 spectrophotometer and a Hitachi 850 spectrofluorometer, respectively. Linear dichroism (LD) spectrum was measured at 6°C by the gel squeezing method using a Hitachi 330 spectrophotometer and glass plate polarizers; concentrations of acrylamide and bisacrylamide were 12 and 0.24%, respectively, and a gel was placed in a custom-made thermostatted cuvette holder [26]. Magnetic circular dichroism (MCD) spectra were measured as described previously [27]. Digitized spectral data were transferred to a microcomputer (HP 216) and processed, that is, the baseline subtraction, correction of the spectral sensitivity of the fluorometer for both emission and excitation spectra [26] and the second derivative spectrum by the method of Savitzky and Golay [28]. A light-induced difference absorption spectrum of Pheo *a* was measured under the presence of sodium dithionite (2 mg/ml) and methyl viologen (2 μM) with a Hitachi 557 dual-wavelength spectrophotometer by using a Corning 4–96 filter. Red light from a slide projector with a 650-W tungsten lamp was illuminated after passing through a Toshiba R-67 filter. Spectra measured under the illuminated and the dark conditions were transferred to a microcomputer, and the difference was calculated. A differential extinction coefficient of Pheo *a* at 545 nm was 8 mM^{-1} per cm [29]. Time-resolved fluorescence spectra at 77 K and analysis of decay kinetics were performed as described previously [30].

3. Results

3.1. Analyses with absorption spectroscopy

Fig. 1 shows the absorption (lower) and its second derivative (upper) spectra at 77 K of the untreated D_1-D_2-cyt b_{559} complex, in which Pheo molecules were almost oxidized. In the *Qy* region (Fig. 1, inset), apparent maxima were found at 677 and 671 nm, and those were resolved to

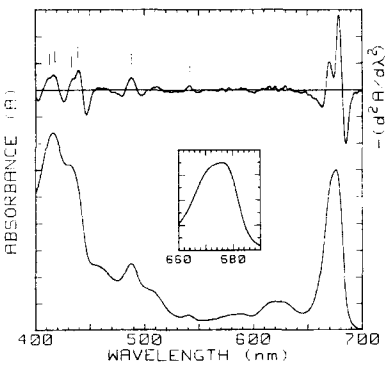

Fig. 1. Absorption and its second derivative spectra of the spinach D_1-D_2-cyt b_{559} complex at 77 K. For the absorption spectrum, samples were dissolved in 50% sodium-glycerophosphate, 25% glycerol, and 25% buffer solution. Second derivative spectrum was obtained by the method developed by Savitzky and Golay [28] and shown after inversion for easy comparison. Small bars on the second derivative spectra indicated the locations of maxima. Inset: A spectrum expanded in the *Qy* region of Chl *a* and Pheo *a*.

679- and 669-nm bands by the derivative spectrum; the former most probably corresponded to P680. These are consistent with the previous reports [15–17,31,32]. In the *Qx* region of Pheo *a*, one maximum was observed around 540 nm with an asymmetric band shape; steep in the long wavelength side. The second derivative spectrum indicated the location of maximum at 542 nm. A band corresponding to β-carotene was also resolved. In the Soret region, plural bands were resolved for both of Pheo *a* (413 and 418 nm) and Chl *a* (433 and 440 nm). Absorbance of cyt b_{559} was estimated to be at most 15% of total absorbance at 415 nm [33] based on the difference spectrum of α-band of cyt b_{559}. The presence of two Soret bands of Pheo *a* was also observed for the complex containing less than 6 Chl *a* (data not shown), suggesting that the presence of plural Pheo *a* bands was an intrinsic property of the complex.

LD and absorption spectra of the complex at 6°C are shown in Fig. 2. In the chl *a Qy* region, a positive peak at 682 nm and a negative peak at 667 nm were clearly resolved, as observed at 10 K [34,35]. In the Soret region, a negative peak was found at 406 nm, and a positive peak, at 418 nm; the latter most probably corresponded to Pheo *a*, however, the former was not clearly assigned. The band corresponding to cyt b_{559} was not resolved by the LD spectrum. In the *Qx* region of Pheo *a*, the peak was located at 543 nm with an asymmetric band shape as in the 77 K absorption spectrum (Fig. 1). Plural Pheo molecules were discriminated by MCD spectrum at 4°C (curve 3 in the inset of Fig. 2); a plateau between 538 and 542 nm indicated the presence of plural forms of Pheo molecules. Two β-carotene bands were clearly resolved; one corresponding to a series of positive peaks (at 443 and 473 nm) and the other, a series of negative peaks (at 429, 458, and 489 nm). Since the energy difference between the two

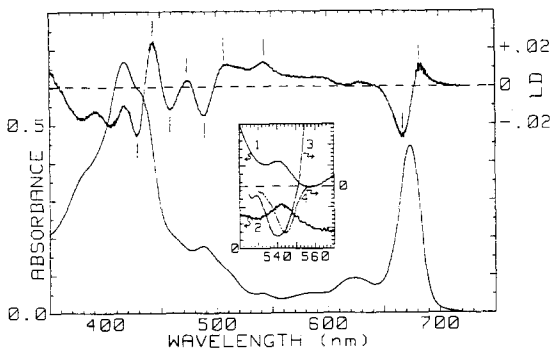

Fig. 2. Absorption and LD spectra of the spinach D_1-D_2-cyt b_{559} complex at 6°C. For LD spectrum, samples were dissolved in 12% acrylamide with a reduced concentration of ammonium persulfate, and acrylamide was polymerized at 4°C, then a gel was compressed from one direction to a half of the original thickness. Inset: (1) absorption, (2) LD, (3) MCD at 4°C, and (4) difference absorption spectra at 5°C induced by the reversible photochemical reduction of Pheo *a*. For the MCD and difference absorption spectra, a reductant, sodium dithionite (2 mg/ml) and a mediator, methyl viologen (2 μM) was added. Difference absorption spectrum was obtained by illumination of red-light with an intensity of 1500 $\mu E/m^2/s$ (see Section 2).

peaks in individual series was nearly equal to 1400 cm^{-1} as known for the S_2 state of carotenoids [36], the third peak for the positive peak series was expected at 507 nm, which was shown by a dotted line (Fig. 2).

Since the Pheo *a Qx* band seemed to consist of plural bands, we identified a phtochemically active Pheo *a* molecule by difference absorption spectrum induced by the photochemical reduction of Pheo *a* at 5°C (Fig. 2, inset). Under the presence of a reductant (sodium dithionite, 2 mg/ml) and a mediator (methyl viologen, 2 μM), light illumination induced the reversible reduction of Pheo *a* with a negative peak at 544.5 nm and a band width of approx. 430 cm^{-1} at $1/e$ of the maximum (curve 4 in Fig. 2 inset). This negative band corresponded to the long wavelength form of Pheo *a* molecules found by the MCD spectrum (curve 3). Compared with the absorption maximum at 6°C (541.5 nm, curve 1 in Fig. 2 inset), this peak was significantly red-shifted, indicating the presence of other component(s) in the short wavelength region of the *Qx* band as observed in the LD spectrum at 6°C (curve 2 in Fig. 2 inset). The presence of two types of Pheo *a* molecules was extensively analyzed by fluorescence spectrum.

3.2. Analyses with fluorescence spectroscopy

The fluorescence emission maximum of the D_1-D_2-cyt b_{559} complex at 77 K was 683 nm (Fig. 3A, solid line), as described in previous reports [14,16,30,37], when Chl *a* was excited at 435 nm. Excitation of Pheo *a* at 415 nm (Fig. 3C) and 540 nm (data not shown) revealed peak wavelengths and spectral shape virtually identical to those obtained by 435 nm excitation.

In contrast, the fluorescence excitation spectra varied depending on the monitoring wavelength (Fig. 4). For example, when the sample was monitored at 740 nm, where the vibrational band of the main emission is located, many peaks were detected (Fig. 4A, a full line). The peaks were assigned to Chl *a* (680, 672, 631, 586, and 435 nm), Pheo *a* (618, 543, 513, and 418 nm), and β-carotene (489 nm). In comparison, the excitation spectrum monitored at 665 nm was very different (Fig. 4B, a full line). Major peaks were found at 414 nm and 435 nm, and minor peaks were found at 537 and 612 nm. The 435-nm band was assigned to Chl *a*, and the remaining three bands were assigned to Pheo *a*. The peak wavelengths of the three Pheo *a* bands were clearly blue-shifted, compared with those detected by monitoring at 740 nm. This difference revealed the presence of a short-wavelength form of Pheo *a* in the isolated PS II RC complex. Two Pheo *a* bands at 414 and 418 nm were consistent with those resolved by the second derivative absorption spectrum (Fig. 1). In addition, a difference was also observed in the wavelength region for β-carotene; monitoring at 665 nm revealed two faint new bands at 506 and 467 nm, instead of the band at 489 nm which had been observed by monitoring at 740 nm.

The photochemical reduction of Pheo *a* induced a significant change in fluorescence properties. The overall fluorescence yield decreased to 21% of that of the Pheo-oxidized samples upon excitation at 435 nm (Fig. 3A, a broken line), consistent with a previous report [31]. In addition, the emission maximum was slightly blue-shifted and the band width was 6-nm wider than that of the Pheo-oxidized samples, as shown by the normalized spectra (Fig. 3B, a broken line), in which three spectra (Pheo-oxidized, Pheo-reduced and the difference between them) were shown after normalization. The emission difference spectrum, which was obtained by subtraction of spectrum of Pheo-reduced sample from that of Pheo-oxidized samples (denoted as ΔF in Fig. 3A), was slightly different from the spectrum of Pheo-oxidized sample (a full line in Fig. 3A); no significant intensity was detected in the wavelength region around 670 nm. On the other hand, the spectrum of the Pheo-reduced sample (Fig. 3A, a broken line) clearly showed the apparent bump around 670 nm, and the fluorescence intensity at 670 nm was almost the same under both the Pheo-reduced and Pheo-oxidized conditions. This component(s) was fluorescent under both conditions, and was insensitive to the Pheo-reduction. The excitation of Pheo *a* at 415 nm (Fig. 3C) and 540 nm (data not shown) yielded similar observations.

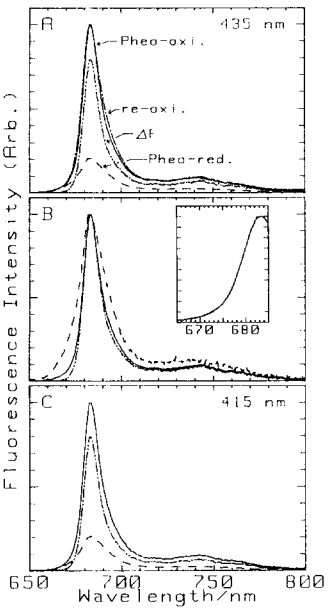

Fig. 3. Corrected fluorescence emission spectra of the spinach D_1-D_2-cyt b_{559} complex at 77 K. Emission spectra were measured by excitation at 435 nm (A and B) and 415 nm (C), and were corrected for the spectral sensitivity of the apparatus. Spectra of the Pheo-oxidized sample are shown by solid lines, those of the Pheo-reduced sample, by broken lines, and difference emission spectra of the Pheo-oxidized and Pheo-reduced sample (ΔF), by alternate lines with two dots. The numbers in the upper right corners denote the excitation wavelengths. In (A), the spectrum of the re-oxidized sample is also shown, and is represented by an alternate line with one dot. In (B), three spectra excited at 435 nm are shown after normalization; the spectrum of the Pheo-oxidized sample, of the Pheo-reduced sample, and of the difference between them. Bandwidth for the excitation was 3 nm and fluorescence was detected with a bandwidth of 1 nm. Inset of (B) indicated the normalized fluorescence spectra of the Pheo-oxidized samples excited at 415 nm (dotted line) and 435 nm (full line). Polyethylene glycol 4000 was added to the sample solution before illumination (at a final concentration of 15%) to facilitate the formation of homogenous ice.

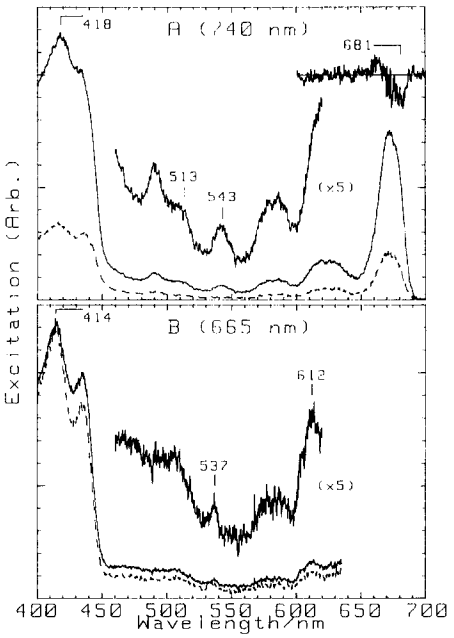

Fig. 4. Corrected fluorescence excitation spectra of the spinach D_1-D_2-cyt b_{559} complex at 77 K. All spectra were corrected for the spectral sensitivity of the apparatus. (A), spectra monitored at 740 nm and (B), those monitored at 665 nm. Spectra of the Pheo-oxidized sample are represented by solid lines and those of the Pheo-reduced sample, by broken lines. In the wavelength region from 460 to 620 nm, a 5-fold magnification of the Pheo-oxidized spectra is shown. The small numbers represent the peak wavelengths of Pheo *a*. In (A), the difference spectrum between the Pheo-oxidized and Pheo-reduced samples, obtained by subtracting the spectra after normalization, is shown in the upper right. The bandwidths of the excitation and emission monochromator were 2 and 3 nm, respectively.

The observed decrease in emission intensity of the Pheo-reduced sample can be attributed to the lack of charge recombination; this is confirmed by the decay kinetics at 683 nm under the Pheo-reduced condition (Fig. 5A, a dotted line), which reveals the absence of a long-lived (39.8 ns [30] or 39.5 ns [38]) fluorescence component. Such observations are consistent with a previous report [39] indicating that the formation of $P680^+$ was not observed under the Pheo-reduced condition. Furthermore, the Pheo-reduced sample showed a slightly slower fluorescence decay at 683 nm in the ns time range than that of the Pheo-oxidized samples (Fig. 5A). Thus it appears that the energy transfer was less efficient in the Pheo-reduced sample, resulting in the fluorescence from individual pigments as shown in Fig. 3B (a broken line). The emission difference spectrum at 77 K (denoted as ΔF in Fig. 3) reveals the delayed fluorescence (DF) from $P680^*$ and from some of the pigments that accept energy from $P680^*$ at 77 K. Consequently, the emission of the Pheo-oxidized

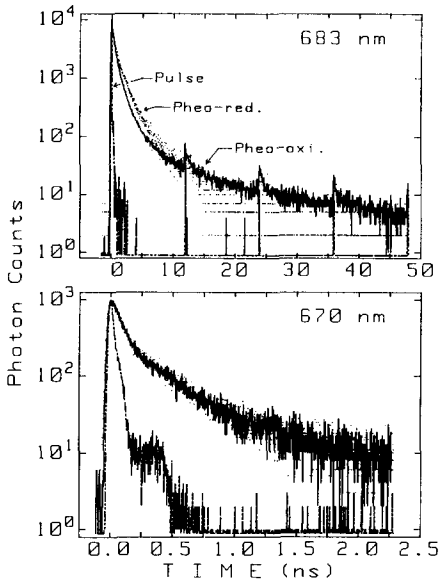

Fig. 5. Decay kinetics of fluorescence at two wavelengths of the spinach D_1-D_2-cyt b_{559} complex at 77 K. In (A), the normalized decay curves at 683 nm measured in a long timescale and (B) those at 670 nm in a short timescale were shown. Full lines indicate the decay curves under the Pheo-oxidized condition and dotted lines, those under the Pheo-reduced condition. Alternate lines show the pulse profile for excitation at 630 nm.

sample was a mixture of the prompt fluorescence (approx. 20%) and the DF (approx. 80%).

The accumulation of $Pheo_{ac}^-$ induced significant changes in the excitation spectrum (Fig. 4). For example, the intensity of the *Qy* band decreased to approx. 25% of the intensity observed in the Pheo-oxidized sample when monitored at 740 nm (Fig. 4A, a broken line), in accordance with a decrease in the emission intensity (Fig. 3A,C, broken lines). In addition, the location of the *Qy* transition revealed a slight blue-shift and the intensity was lower at 543 nm than that of the Pheo-oxidized samples. Furthermore, peaks were observed at 418, 436, 670, and 677 nm. The difference excitation spectrum which represents the components responsible for charge recombination and thus separation, was essentially similar to the spectrum of the Pheo-oxidized sample (data not shown). When the excitation spectra for prompt fluorescence (Pheo-reduced) and total fluorescence (Pheo-oxidized) were normalized and compared, there was a detectable difference in the contribution of individual pigments induced by Pheo-reduction. The difference maximum (Fig. 4A, upper right) was observed at 681 nm, which is consistent with bleaching of the $Pheo_{ac}$ molecule. Thus, we can conclude that $Pheo_{ac}$ has an absorption band at 681 nm, which overlaps that of P680.

In contrast to the observations at 740 nm, the accumulation of $Pheo_{ac}^-$ did not appear to affect the excitation spectrum of the sample monitored at 665 nm (Fig. 4B, a broken line). Bands corresponding to Pheo *a* were observed at 414, 537, and 612 nm and had almost the same intensities as those of the Pheo-oxidized sample; in addition, the contribution of Chl *a* decreased to some extent. The decrease indicates that the accumulation of $Pheo_{ac}^-$ did not influence the fluorescence properties of the short wavelength form of Pheo *a*.

In order to identify the origin of fluorescence, we measured fluorescence decay curves at two wavelengths and estimated lifetimes of components. At 683 nm, a long-lived component was clearly observed in the Pheo-oxidized condition (Fig. 5A, a full line); since this component was absent in the Pheo-reduced condition (Fig. 5A, a dotted line), this was assigned to the fluorescence due to a charge recombination between $P680^+$ and $Pheo_{ac}^-$. At 670 nm, the effect of Pheo-reduction was insignificant (note that the time-scale was different between Fig. 5A and 5B); the decay curves were essentially the same, irrespective of the redox conditions of Pheo *a* up to 50 ns time range (data not shown). Fluorescence lifetimes were estimated by the convolution calculation with the assumption of exponential decay function [30]. The long-lived component responsible for the charge recombination showed its decay time of 39.8 ns. Other components at 683 nm under the Pheo-oxidation condition were 30.5 ps, 598 ps, and 2.87 ns (Table 1). On the contrary, under the Pheo-reduced condition, three components were enough for the best fit of the decay at 683 nm; those were 133 ps, 1.24 ns, and 6.13 ns. The presence of a component with a lifetime of 1 ns range was already reported [30]. At 670 nm, the decay curves were reasonably fit by three components which were essentially identical under the Pheo-oxidized and Pheo-reduced condition (Table 1). A long-lived component was

Table 1
Estimation of fluorescence lifetimes in the D_1-D_2-cyt b_{559} complex at 77 K

	A_1	τ_1 (ps)	A_2	τ_2 (ps)	A_3	τ_3 (ps)	A_4	τ_4 (ns)
Pheo-oxi.								
670 nm	0.836	40.3	0.127	214	0.037	646		
683 nm	0.826	30.5	0.160	598	0.012	2870	0.002	39.8
Pheo-red.								
670 nm	0.777	33.6	0.179	166	0.044	662		
683 nm	0.723	133	0.267	1240	0.010	6130		

A and τ mean amplitudes and lifetimes, respectively. An exponential function was assumed for the fluorescence decay in each calculation. For details in the experiments, see Ref. [30].

absent in the short wavelength region irrespective of the redox state of Pheo *a*, consistent with the fluorescence spectrum (Fig. 3A). A lifetime corresponding to a free pigment (6–8 ns for Pheo *a* and 6 ns for Chl *a*, see Table 1 in Ref. 39) was substantially undetected in the wavelengths from 665 to 690 nm. Even if the component(s) whose lifetime was longer than 2 ns was regarded as a free or disintegrated component(s), its fraction was less than 2%, clearly indicating that free pigment(s) was virtually absent in our preparation.

The fluorescence properties of the Pheo-reduced sample returned to the original state following the oxidation of Pheo *a*. When the reduced sample was warmed up to 4°C, stored in the dark for 5 min, and then frozen without exposure to light, the fluorescence spectrum and quantum yield (Fig. 3A, denoted as re-oxi.) was essentially identical to those of the Pheo-oxidized sample. Furthermore, the *Qx* band of Pheo *a* was observed at 543 nm when the excitation spectrum was monitored at 740 nm (data not shown). These results indicate that changes in the fluorescence properties induced by Pheo *a* reduction were reversible, and that they were not caused by destructive effect due to Pheo-reduction, but reflected changes in the redox state of the primary electron acceptor.

Our results indicate that there are two, spectrally distinguishable, Pheo *a* molecules. One of the molecules has peaks at 681, 618, 543, 513, and 418 nm, and the other has peaks at 612, 537, and 414 nm. The former corresponds to $Pheo_{ac}$ [10,14,15,21,41], and the latter to $Pheo_{iac}$. The location of the *Qy* band of the latter will be discussed below.

4. Discussion

Optical properties of Pheo *a* in organic solvents vary to some extent depending on solvents; in many cases, absorption maxima are located at 669 ± 3 nm (*Qy*), 538 ± 3 nm (*Qx*) and 412 ± 3 nm (Soret) [24,42], and a fluorescence lifetime is in a range of 6 to 8 ns with a fluorescence quantum yield of approx. 0.2 (see Table 1 in Ref. 39). Since the locations of *Qx* and Soret bands of $Pheo_{iac}$ we observed were close to those in organic solvents, it is crucial to exclude the possibility of contamination of free Pheo *a* and also free Chl *a*.

It is known that the optical properties of the D_1-D_2-cyt b_{559} complex strongly depend on detergents [31,43–45]. The 683 nm fluorescence maximum is possibly attributed to the blue-shifted peak due to a remaining unfavorable detergent, i.e., Triton X-100 [31,44]. We therefore investigated the effect of Triton X-100 on our preparation. A very low concentration (0.05%) of Triton induced an increase in absorbance of at the most 2% at 670 nm after 10 min incubation at 4°C. Fluorescence maximum at 77 K was kept at 683 nm; however, a new band appeared at 670 nm with an increase in a fluorescence yield by 30 times, which was easily detected by fluorescence decay kinetics. As shown in Fig. 5 and Table 1, this was not the case with our original preparation. Furthermore, the time-resolved fluorescence spectrum in the ns range of the Pheo-oxidized sample indicated the presence of DF at 683 nm (for example, see Fig. 3 in Ref. 27); the main emission was a single Gaussian band with a full bandwidth of ca. 160 cm^{-1} (data not shown). This band shape is consistent with the 683 nm steady-state fluorescence band observed in the difference fluorescence spectra (ΔF in Fig. 3). If the 683 nm band originates from an antenna pigment which equilibrates with the $P680^*$ at 77 K, plural DF bands from an antenna and $P680^*$ can be expected, whose relative intensities follow the Boltzmann distribution. This was not observed in our preparation.

In our preparation, approx. 47% of total Pheo *a* was photochemically and reversibly reduced at 20°C (data not shown), indicating that one molecule out of two Pheo *a* was $Pheo_{ac}$ as indicated previously [10]. When the fluorescence spectra excited at 415 nm and 435 nm were normalized and compared, the spectra were identical within an experimental error of 2% (Fig. 3B, inset), strongly suggesting the absence of free Pheo *a* in our preparation. Similarly, a long-lived fluorescence component was scarcely observed in the decay curve at 670 nm (Fig. 5 and Table 1), where an emission of the free Pheo *a* was expected. Since free Chl *a* has a lifetime of about 6 ns in organic solvents [39] and in the complex [40], the presence of free Chl *a* was also less probable. Recently, Mishra et al. [45] reported that a short wavelength form of Pheo *a* molecule was formed when PS II core complex was damaged by photoinhibition. However, this is not applicable to our case, because two Pheo *a* bands were always observed irrespective of the redox condition of Pheo *a*, and changes in the fluorescence properties were reversible, as shown in Fig. 3A. Since it is known that several fluorescence components are present in our preparation [30], the fluorescence monitored at 665 nm corresponded to a short wavelength component(s) in this complex, and might not be due to the damaged samples. Contamination of free pigments was estimated to be at most 2% of total pigments from the fluorescence lifetimes (Fig. 5 and Table 1). Based on these observations, we assigned the short-wavelength form of Pheo *a* to $Pheo_{iac}$. Furthermore, we now think that the proportion of DF to the total fluorescence (approx. 80% in our preparations) under the Pheo-oxidized condition and similarity of spectra between the DF and the total fluorescence can be indices for intactness of preparations, as well as the peak position as discussed previously [20,31,44].

In the present study, we found two forms of the Pheo *a* molecule, $Pheo_{ac}$ and $Pheo_{iac}$, in the D_1-D_2-cyt b_{559} complex based on the spectral peaks of *Qx* and Soret bands in the fluorescence excitation spectra. Despite this information, the location of the *Qy* band of $Pheo_{iac}$ could not be predicted directly from the spectrum. However, the energy difference between $Pheo_{iac}$ and $Pheo_{ac}$ can be determined and was estimated to be 206 cm^{-1} (537 versus 543 nm)

for the *Qx* band and 231 cm^{-1} (414 versus 418 nm) for the Soret band. If we then apply a similar band-shift magnitude to the *Qy* band, the *Qy* maximum of $Pheo_{iac}$ at 77 K can be predicted to be 671 ± 1 nm, based on the peak position of the *Qy* band of $Pheo_{ac}$, which is at 681 nm (Fig. 4A). It may be coincidence that locations of absorption bands of $Pheo_{iac}$ are similar to those of free Pheo *a*.

Two forms of BPheo have also been found in *Rhodopseudomonas viridis*, the $BPheo_{ac}$ molecule has *Qy* and *Qx* bands at 810 and 545 nm, respectively, whereas $BPheo_{iac}$ has absorption maxima at 790 and 530 nm [46]; only the long wavelength form of BPheo is photochemically active. The same tendency was observed in RC complex isolated from *Rhodopseudomonas palustris*; (530 ± 1 nm for $BPheo_{iac}$ and 541 ± 1 nm for $BPheo_{ac}$, Mimuro et al., unpublished). In the present study, the PS II RC exhibits the same feature, confirming a similarity in the pigment organization of the PS II RC and the purple bacterial RC.

The *Qx* absorption band of Pheo *a* in the spinach D_1-D_2-cyt b_{559} complex has never been resolved to plural bands even at 6 K [22]. This was also the case of LD spectrum at 10 K [34,35]. This may be due to a small difference in the locations of two bands in the D_1-D_2-cyt b_{559} complex (ca. 200 cm^{-1}), contrary to the case of *R. viridis* (ca. 520 cm^{-1}). Our observation by absorption (Fig. 2), LD (Fig. 2), and circular dichroism (data not shown) did not necessarily give a fair basis for the presence of plural bands. On the other hand, MCD spectrum (Fig. 2, inset) clearly indicates the plural forms. Compared with the other methods, MCD spectrum is more sensitive to Pheo *Qx* band [47]. In the previous paper [27], we simulated the MCD *Qx* band of Pheo *a* by a single component; however, now it became clear that this *Qx* band should be simulated by two components having bandwidths of 540 cm^{-1} (for $Pheo_{ac}$) and 340 cm^{-1} (for $Pheo_{iac}$) (data not shown). A wider bandwidth of the $Pheo_{ac}$ (Fig. 4A) was also unfavorable for detection of the $Pheo_{iac}$ which has a narrower bandwidth (Fig. 4B). Recently, Chang et al. [22] reported that the asymmetric absorption band of Pheo *a* in the complex containing 6 Chl *a*; the bandwidth was wider in the short wavelength region. This result can be interpreted as showing that an additional band was present in the short wavelength region, which is consistent with our observation. The extinction coefficients of two Pheo *a* molecules were not definitely determined by the excitation spectrum. A relative intensity of the *Qx* band to that of Soret band was very similar between $Pheo_{ac}$ and $Pheo_{iac}$ (Fig. 4). However, contribution of $Pheo_{iac}$ to the total absorption was estimated to be smaller, because apparent absorption properties of the complex were rather similar to those of $Pheo_{ac}$. This might be the second reason why the two Pheo *a* bands have not been clearly resolved.

An extremely low fluorescence yield was observed for $Pheo_{iac}$ (Fig. 3), indicating that the pigment is involved in the energy transfer sequence in the RC complex, thus the molecular interaction between pigments should be intact in the isolated PS II RC. The low fluorescence yield may also explain why $Pheo_{iac}$ was not detected by fluorescence spectroscopy in earlier studies. In the bacterial RC, $BPheo_{iac}$ is postulated to be coupled with other pigments in terms of electronic state [48] and P^* is assigned to be a quencher of fluorescence [49]. These characteristics have not yet been elucidated in the PS II RC complex. Three previous studies have reported the presence of an upper exciton band of the special pair in the PS II RC complex [9,15,22], but the band could not be resolved in the present analysis. The molecular structure of the special pair has been reported to be somewhat distorted in the isolated complex [4–9], thus the presence of an upper exciton band of the special pair may be unclear.

There have been a few reports suggesting the presence of two kinds of β-carotene molecules in the D_1-D_2-cyt b_{559} complex, as determined by spectral [16,35] and photochemical [50] data. The LD (Fig. 2) and fluorescence excitation spectra (Fig. 4) in the present study also revealed two types of β-carotene, confirming the previous reports. One type is associated with P680 and may correspond to the β-carotene molecule which is bleached in accordance with the 670-nm Chl *a* (accessory Chl *a*) [31,50]. The other type was detected in the excitation spectrum monitored at 665 nm and may be associated with $Pheo_{iac}$ and/or Chl *a*, which emits in the region of 665 nm, although there is no direct evidence to indicate that the second type of β-carotene is coupled with $Pheo_{iac}$. One hypothesis, that there is an exciton interaction between two β-carotene molecules [50], does not appear to be possible; two independent excitation spectra were observed in the present study (Fig. 4).

The molecular arrangement of pigments in the PS II RC has been postulated to be analogous to that of purple bacterial RC, based on the similarity of amino acid sequence in the constituent protein subunits and the deduced protein structures [1–3]. The $BPheo_{ac}$ molecule resides on the L-subunit, and $BPheo_{iac}$ molecule is on the M-subunit in the bacterial RC. Therefore, in PS II RC the $Pheo_{ac}$ molecule is probably located on the D_1 polypeptide and the $Pheo_{iac}$ molecule, on the D_2 subunit. In addition, the β-carotene that functions as a triplet quencher in conjunction with accessory Chl *a* is thought to reside on the D_2 subunit, as in bacterial RC. However, the location of the other β-carotene, which may couple with a short-wavelength form of Chl *a* and/or $Pheo_{iac}$, is unknown. The localization of this β-carotene molecule and the additional two Chl *a* molecules, will be the subject of our next study.

Acknowledgements

We would like to thank Dr. M. Kobayashi, Tsukuba University, for his suggestion regarding the determination

of pigment content, and to Prof. T. Nozawa, Drs. M. Kobayashi, and Z.-Y. Wang, Tohoku University for their help for MCD spectroscopy. This work was supported, in part, by a Grant-in-Aid for Co-operative Research (04304004) from the Ministry of Education, Science, Sport and Culture, Japan to K.S. and M.M., and a Priority Area grant (04273101) to K.S. This work was also supported by a grant from the Human Frontier Science Program to K.S. T.T. is a recipient of a pre-doctoral fellowship from JSPS. M.M. also thanks the Ciba-Geigy Foundation (Japan) for the Promotion of Science for the financial support.

References

[1] Satoh, K. (1993) in The Photosynthetic Reaction Center (Deisenhofer, J. and Norris, J.R. eds.), vol. 1., pp. 289–318, Academic Press, San Diego.
[2] Deisenhofer, J. and Michel, H. (1989) EMBO J. 8, 2149–2170.
[3] Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1985) Nature 318, 618–624.
[4] Lösche, M., Satoh, K., Feher, G. and Okamura, M.Y. (1988) Biophys. J., 53, 270a.
[5] Van Mieghem, F.J.E., Satoh, K. and Rutherford, A.W. (1991) Biochim. Biophys. Acta 1058, 379–385.
[6] Noguchi, T., Inoue, Y. and Satoh, K. (1993) Biochemistry 32, 7186–7195.
[7] Van Dorssen, R.J., Breton, J., Plijter, J.J., Satoh, K., Van Gorkom, H.J. and Amesz, J. (1987) Biochim. Biophys. Acta 893, 267–274.
[8] Newell, W.R., Van Amerongen, H., Barber, J. and Van Grondelle, R. (1991) Biochim. Biophys. Acta 1057, 232–238.
[9] Otte, S.C.M., Van der Vos, R. and Van Gorkom, H.J. (1992) J. Photochem. Photobiol. B 15, 5–14.
[10] Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
[11] Akabori, K., Tsukamoto, H., Tsukihata, J., Nagatsuka, T., Motokawa, O. and Toyoshima, Y. (1988) Biochim. Biophys. Acta 932, 345–357.
[12] Kobayashi, M., Maeda, H., Watanabe, T., Nakane, H. and Satoh, K. (1990) FEBS Lett. 260, 138–140.
[13] Montoya, G., Yruela, I. and Picorel, R. (1991) FEBS Lett. 283, 255–258.
[14] Yruela, I., van Kan, P.J.M., Müller, M.G. and Holzwarth, A.R. (1994) FEBS Lett. 339, 25–30.
[15] Van Kan, P.J.M., Otte, S.C.M., Kleinherenbrick, F.A.M., Nieveen, M.C., Aatrsma, T.J. and Van Gorkom, H.J. (1990) Biochim. Biophys. Acta 1020, 146–152.
[16] Kwa, S.L.S., Newell, W.R., Van Grondelle, R. and Dekker, J.P. (1992) Biochim. Biophys. Acta 1099, 193–202.
[17] Montoya, G., Cases, R., Yruela, I. and Picorel, R. (1993) Photochem. Photobiol. 58, 724–729.
[18] Van der Vos, R., Van Leeuwen, P.J., Braun, P. and Hoff, A.J. (1992) Biochim. Biophys. Acta 1140, 184–198.
[19] Van der Vos, R. and Hoff, A.J. (1995) Biochim. Biophys. Acta 1228, 73–85.
[20] Braun, P., Greenberg, B.M. and Scherz, A. (1990) Biochemistry, 29, 10376–10387.
[21] Tang, D., Jankowiak, R., Seibert, M., Yocum, C.F. and Small, G.J. (1990) J. Phys. Chem. 94, 6519–6522.
[22] Chang, H.-C., Jankowiak, R., Reddy, N.R., Yocum, C.F., Picorrel, R., Seibert, M. and Small, G.J. (1994) J. Phys. Chem. 98, 7725–7735.
[23] Tomo, T. and Satoh, K. (1994) FEBS Lett. 351, 27–30.
[24] Watanabe, T., Hongu, A., Honda, K., Nakazato, M., Konno, M. and Saitoh, S. (1984) Anal. Chem. 56, 251–256.
[25] Davies, B.H. (1976) in Chemistry and Biochemistry of Plant Pigments (Goodwin, T.W. ed.), 2nd ed., Vol. 2, pp. 38–165, Academic Press, London.
[26] Mimuro, M., Katoh, T. and Kawai, H. (1990) Biochim. Biophys. Acta 1015, 450–456.
[27] Nozawa, T., Kobayashi, M., Wang, Z.-Y., Itoh, S., Iwaki, M., Mimuro, M. and Satoh, K. (1995) Spectrochim. Acta 51A, 125–134.
[28] Savitzky, A. and Golay, M.J.E. (1964) Anal. Chem. 36, 1627–1639.
[29] Goedheer, J.C. (1966) in The Chlorophylls (Vernon, L.P. and Seely, G.R. eds.), pp. 147–184, Academic Press, New York.
[30] Mimuro, M., Yamazaki, I., Itoh, S., Tamai, N. and Satoh, K. (1988) Biochim. Biophys. Acta 933, 428–436.
[31] Tetenkin, V.L., Gulyaev, B.A., Seibert, M. and Rubin, A.B. (1989) FEBS Lett. 250, 459–463.
[32] Angerhofer, A., Friso, G., Giacometti, G.M., Carbonera, D. and Giacometti, G. (1994) Biochim. Biophys. Acta 1188, 35–45.
[33] Wasserman, A.R., (1980) in Methods in Enzymology, Vol. 69 (San Pietro, A. eds.), pp. 181–203, Academic Press, New York.
[34] Satoh, K. (1993) in Frontiers of Photobiology (Shima, A., Ichihashi, M., Fujiwara, Y. and Takabe, H. eds.), pp. 3–11, Excerpta Medica, Amsterdam.
[35] Breton, J., Duranton, J. and Satoh, K. (1988) in Photosynthetic Light-Harvesting Systems: Organization and Function (Scheer, H. and Schnedier, S. eds.), pp. 375–386, Walter de Gruyter, Berlin.
[36] Mimuro, M., Nagashima, U., Nagaoka, S., Takaichi, S., Yamazaki, I., Nishimura, Y. and Katoh, T. (1992) Chem. Phys. Lett. 204, 101–105.
[37] Freiberg, A., Timpmann, K., Moskalenko, A.A. and Kuznetsova, N.Y. (1994) Biochim. Biophys. Acta 1184, 45–53.
[38] Roelofs, T.A., Kwa, S.L.S., Van Grondelle, R., Dekker, J.P. and Holzwarth, A.R. (1993) Biochim. Biophys. Acta 1143, 147–157.
[39] Wasielewski, M.R., Johnson, D.G., Seibert, M. and Govindjee (1989) Proc. Natl. Acad. Sci. USA 86, 524–528.
[40] Seely, G.R. and Connolly, J.S. (1986) in Light Emission by Plants and Bacteria (Govindjee, Amesz, J. and Fork, D.C. eds.), pp. 99–133, Academic Press.
[41] Hastings, G., Durrant, R., Barber, J., Porter, G. and Klug, D.R. (1992) Biochemistry 31, 7638–7647.
[42] Vernon, L.P. (1960) Anal. Chem. 32, 1145–1150.
[43] Crystall, B., Booth, P.J., Klug, D.R., Barber, J. and Porter, G. (1989) FEBS Lett. 249, 75–78.
[44] Seibert, M., Picorel, R., Rubin, A.B. and Connolly, J.S. (1988) Plant Physiol. 87, 303–306.
[45] Mishra, N.P., Francke, C., Van Gorkom, H.J. and Ghanotakis, D.F. (1994) Biochim. Biophys. Acta 1186, 81–90.
[46] Shuvalov, V.A., Shkuropatov, A.Y. and Ismailov, M.A. (1987) In Progress in Photosynthesis Research (Biggins, J., ed.), vol. 1, pp. 161–168, Martinus Nijhoff, Dordrecht.
[47] Houssier, C. and Sauer, K. (1970) J. Am. Chem. Soc. 92, 779–791.
[48] Parson, W.W. and Warshel, A. (1993) in The Photosynthetic Reaction Center (Deisenhofer, J. and Norris, J.R., eds.), Vol. 2., pp. 23–47, Academic Press, San Diego.
[49] Parson, W.W., Clayton, R.K. and Cogdell, R.J. (1975) Biochim. Biophys. Acta 387, 265–278.
[50] Rivas, J.D.L., Telfer, A. and Barber, J. (1993) Biochim. Biophys. Acta 1142, 155–164.